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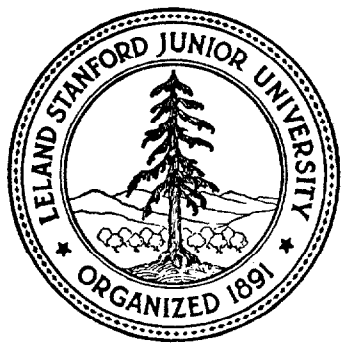
CYTOCHEMICAL STUDIES OF PLANETARY MICROORGANISMS EXPLORATIONS IN EXOBIOLOGY

Status Report Covering Period January 1, 1971 to December 31, 1971
For
National Aeronautics and Space Administration
Grant NGR-05-020-004



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Report to the National Aeronautics and Space Administration

"Cytochemical Studies of Planetary Microorganisms - Explorations in Exobiology"

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Instrumentation Research Laboratory, Department of Genetics

Stanford University School of Medicine

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A. INTRODUCTION

This report covers the activities of the Instrumentation Research Laboratory during the calendar year January 1, 1971 to December 31, 1971.

While the main support of the IRL activities during this period continued to be the NASA grant NGR-05-020-004, some of the funds have come from other grants, other agencies, and in some cases private institutions. This report includes all the activities of the laboratory which relate to or have benefited from NASA support regardless of whether or not they were primarily supported by this NASA grant.

The Dendral project receives direct support from NIH grant GM 00612-01 and indirectly from the ACME grant NIH RR 00311-06 and Professor Feigenbaum's ARPA grant SD-183.

Our work in the area of cell separation was supported in this period by NIH grant GM 17367 and NIH contract 69-2064. We received some support for Dr. Duffield's work on the interaction of chlorine compounds with DNA under NIH grant AM 12797 and are seeking additional support for this effort.

The efforts on image processing for the 1971 Mars Mariner are a joint effort with the Artificial Intelligence Laboratory of the Computer Science Department under Professor John McCarthy. It receives support from NASA grant NGR-05-020-508 and JPL Contract 952489. Our work on lander camera imagery receives support under Langley contract NAS 1-9682.

These interrelationships benefit our NASA program in two ways. They aid the rapid utilization for medicine, and biology in general, of the ideas and skills developed because of our interests in space missions. They not only provide us with the opportunity of testing instrumentation methods, applicable to future NASA programs, in circumstances of solving current scientific problems but also provides much needed additional support. This support hopefully will permit the Instrumentation Research Laboratory to continue to function with sufficient technological depth and breadth to allow responses to future NASA needs.

For these reasons we have always felt it desirable to carry out some laboratory work that could lead to new and fruitful relationships. For example, we have collaborated with Professor Marvin Chodorow of the Applied Physics Department on a preliminary evaluation of the application of microwave acoustic scattering to problems of cell discrimination.

The general areas of the Program Resume, Part B of the report, are:

I. Dendral

II. Constituents of Urine

III. Applications of Gas Chromatography

IV. Chlorination of DNA Bases

V. Mass Spectrometry of Organic Compounds

VI. Analysis of Natural Products by Mass Spectrometry

VII. Mariner Mars 1971 Orbiter Photography

VIII. Viking Lander Imagery Investigation

IX. Cell Separation

B. PROGRAM RESUME

I. DENDRAL

This section is a general review of what is known as the Dendral research project. It is presented in three parts; a) Dendral Chemistry, b) Computer Science, and c) Computer Aided Research Instrumentation.

a. Dendral Chemistry

Historically and in summary the Dendral algorithm was developed by Lederberg[1-4] for the enumeration of all the possible isomers of a given empirical composition. The atomic connectivity is represented by a linear notation and the format itself contains the information required to rank the linear formulas for a set of isomers in a canonical dictionary order. Provision exists for the exclusion from the output of chemically unstable (or otherwise undesirable) structures by placing that subgroup of atoms on a Badlist. This list is referenced during structure generation and all structures containing the Badlist subgroup are not produced. Conversely, if only one functional group (or a specified number of such groups) is required then this subgroup is placed on Goodlist and only those structures containing this entity are constructed by the program.

A computer program, Heuristic Dendral, was developed for the computer interpretation of aliphatic ketone mass spectra.[5] This program consisted of several distinct sections. First, the Preliminary Inference Maker, whose function was to examine the low resolution mass spectrum of an unknown aliphatic ketone to deduce whatever portions of the structure of the unknown were possible. This was achieved by presenting this section of the program with a theory of the fragmentation processes of aliphatic ketones in a mass spectrometer. The results of this survey of the data are then presented to a structure generator (the Dendral Algorithm mentioned above) where all possible chemical structures which are consistent with the mass spectrum are enumerated. Those structures which are inconsistent with a second order theory of mass spectrometry are then removed. This was achieved using a predicted mass spectrum for every candidate and comparing this predicted spectrum with the original unknown. Any significant differences between the two mass spectra result in the removal of that candidate structure from further consideration. Finally each of the remaining structures were ranked by a scoring function on the basis of the closeness of fit between the predicted and unknown mass spectra. This resulted in an ordered list of plausible molecules as a solution for the unknown mass spectrum.

Our results, gathered from a series of aliphatic ketone mass spectra, indicated that the correct solution was invariably ranked either first, or in some rare instances, second. These results provided the impetus for further development of the Heuristic Dendral program, and for the next task, domain aliphatic ethers[6] were chosen. The program worked satisfactorily with this class of compound as shown from the output

lists of structures. The correct answer to a particular problem always appeared in the output, generally as the first or second choice of the program.

The program was then expanded to accomplish the interpretation of alphatic amine mass spectra.[7] This represented an added complexity insofar as the number of amines of a given carbon content is considerably larger than either the corresponding ethers or ketones. In other words the total search space the program had to encounter is significantly enlarged.

The next logical extension of the Heuristic Dendral project was in the interpretation of mass spectra generated by the general class of compounds represented by I.[8]

R-X-R'

I, X = O (ethers and alcohols)
X = N (amines)
X = S (thioethers and thiols)

Confronted with an unknown mass spectrum, the program was capable of deciding to which of these class of compounds the unknown belonged. The required heuristics were then employed to identify the precise compound which had produced the mass spectrum. Using nuclear magnetic resonance data, the program invariably arrived at only one solution per problem, viz the correct answer. Without this spectral evidence (i.e. using mass spectrometry alone) the number of possible answers was reduced from many thousands (often millions) to a few hundred. The speed with which the program operated (of the order of 20 spectra per

minute) made it superior to the human chemist in the interpretation of mass spectra in the limited area of aliphatic ethers, thioethers, amines, alcohols and thiols.

A review of the development of the Dendral Algorithm and Heuristic Dendral to this point has been published.[9]

b. Computer Science

The close interaction between computer scientists, chemists, and biomedical scientists and engineers has always been a unique feature of the Dendral project. In the last year cooperating scientists have pushed development of the computer programs in two directions of interest to the computer science community: (I) Application of Artificial Intelligence and (II) Theory Formation by Computer.

As an application, the artificial intelligence work is invested with a richness of concept and organization that is a direct consequence of the requirement to attend to the detail of a real physical problem - and that is lacking in most other A.I. studies that use "toy" problem environments. Because of this richness of the problem environment, and the success of Dendral in solving real problems, the effort is the focus of much attention and comment in the A. I. community.

In theory formation, the Meta-Dendral program has the potential for greatly enhancing the power of mass spectrometry (and perhaps other instrumental methods) as a research resource. A successful Meta-Dendral program will be a "resource amplifier" in the link between mass spectral data and useable scientific knowledge.

1. Applications of Artificial Intelligence to Mass Spectrometry

Objectives:

The overall objective of this part of the research program is to extend the Heuristic Dendral program to analysis of the mass spectra of complex organic molecules. The following specific objectives, which now rest on a firm foundation of experience through our efforts on the steroidal hormones in the estrogen family, can be identified:

(A) Generalization of the programming techniques to make them compound class independent as far as possible.

(B) Incorporation of the cyclic generator into existing algorithms to expand the capabilities of structure and/or substructure generation.

(C) Refinement of planning rules to minimize the number of molecular structures considered by the Dendral algorithm.

(D) Exploitation of the information contained in ancillary mass spectrometric techniques, such as metastable ion spectra, low ionizing voltage spectra and mass spectral pattern shifts in isotopically labeled molecules, for

inclusion in Dendral.

(E) Structuring of the programs to allow use of information obtained from other spectroscopic or chemical techniques.

(F) Use of ACME facilities for the complete integration of the presently separated tasks of data acquisition and data analysis.

Progress:

Considerable progress has been made in the structural analysis of estrogenic steroids by Heuristic Dendral. The results obtained to this date are important from two standpoints. Firstly, this class of molecules represents the first instance of application of the problem solving capabilities of artificial intelligence to biologically important molecules. Secondly, the initial success of this approach for estrogens is encouraging as it has important implications for extension of the method to other complex molecular systems. A paper describing this program has been submitted for publication.*

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*"Applications of Artificial Intelligence for Chemical Inference. VII. An Approach to the Computer Interpretation of the High Resolution Mass Spectra of Complex Molecules. Structure Elucidation of Estrogenic Steroids." D. H. Smith, B. G. Buchanan, R. S. Engelmore, A. M. Duffield, A. Yeo, E. A. Feigenbaum, J. Lederberg and C. Djerassi. Submitted for publication in J. Am. Chem. Soc. (1972).

The operation of the program described in the paper may be briefly summarized. Given the basic estrogen skeleton (without substituents), information on fragmentation processes relevant to estrogens, metastable information and a complete high resolution mass spectrum, Part I of the program, termed analyzer, identifies molecular ions, determines the numbers of various substituents that must be placed on the skeleton, performs the fragmentations for a given placement and searches the mass spectral data for peaks supporting this placement. Part II of the program, termed synthesizer, attempts to reconstruct plausible molecular structures to fit the conclusions of Part I. Part III of the program allows input of rules or chemical knowledge derived from other techniques to search through the Part II results for a most plausible molecular structure.

As the performance of the existing estrogen analysis program has improved through a continuing feedback mechanism whereby results are examined and programs revised as required, certain facts have become apparent that bear directly on the specific objectives (A-F) mentioned above. For example, it is now apparent that both the analyzer and the synthesizer can be structured to be independent of the specific compound class treated by the program, with changes necessary only in the input data to the analyzer mentioned above. Restructuring of these routines, now largely accomplished, will result in a much more general program that can easily be utilized for other classes of organic compounds.

Parts II and III of the program are formally equivalent to a cyclic structure generator, although on a much more simplistic level. The present cyclic generator is nearing completion, and efforts have been made to ensure a relatively easy incorporation of this more rigorous approach into the existing program.

As other classes of complex molecules are examined by these techniques, planning rules will be necessary to provide information on which compound class may be represented by a given mass spectrum. This topic has received some attention, but much more needs to be done.

Metastable ion spectra are discussed in detail in the subsequent section, so that it is sufficient to say that some of this information can presently be utilized by the program to rationalize the spectra of mixtures of compounds to determine the structure of each component. Incorporation of isotopic labeling information is now underway in a preliminary form, using deuterium labeling to identify the number of labile hydrogen atoms (e.g., O-H functionalities) associated with each molecular ion.

Part III is presently being written to provide the capability of facile input of other chemical or spectroscopic information with which to test plausible structures. This includes, for example, the above deuterium labeling information. It also includes 'natural' rules such as the expectation of an oxygen functionality at C-3 and C-17 in the case of estrogens.

The LISP programming language has been successfully implemented on the IBM 360/50 at the ACME Computing Facility by R. I. Berns, who co-authored and maintains Stanford 360-LISP. The full capabilities of LISP are now available to all users of the ACME Facility. Existing ACME programs for acquisition and reduction of the high resolution mass spectral data have been improved over the previous period to ensure that high quality data are available for use by the Dendral (LISP) algorithm.

Plans:

Many of the plans are implicit in the above discussion of objectives and progress. To summarize, it is planned to continue work on the generalization of the Dendral algorithm, used successfully for estrogens, and begin the study of other biologically and pharmacologically important molecules, e.g., progesterone and perhaps cortico-steroids. The cyclic generator will be implemented on its completion. The inclusion of information from other techniques, e.g., isotopic labeling metastable spectra, ^{13}C NMR, will be refined considerably from its present, preliminary state. It is hoped to implement the Dendral algorithm on ACME soon, so that the complete system is resident on one computer, and to begin evaluating the performance of the system in semi-real-time as opposed to present batch processing of the data.

2. Extending the Theory of Mass Spectrometry by a Computer (Meta-DENDRAL)

Objectives:

The broad objective of Meta-Dendral is to study theory formation in science. Previous work on Heuristic Dendral has given us enough experience with writing computer programs which work within the theory of mass spectroscopists who must formulate new sets of mass spectrometry rules for each new class of molecules under consideration. There are many gaps in current textbooks because of the relatively small number of classes of molecules which have been studied systematically by mass spectroscopists. If the computer system can aid in the process of filling those gaps, then it will have advanced the goals of this project by extending the reasoning power of the Heuristic Dendral System. In addition, it is possible that the program could advance the state of knowledge of mass spectrometry.

Progress:

Substantial progress has been made toward Meta-Dendral goals. A detailed research plan has been formulated which makes explicit the nature of the computer programs that need to be written, and the ways this work will build on previous Heuristic Dendral work. The research plan was elaborated in a paper presented to the second International Joint Conference on Artificial Intelligence (London, September 1970).*

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*"A Heuristic Programming Study of Theory Formation in Science" by B. G. Buchanan, E. A. Feigenbaum, J. Lederberg. Stanford Artificial Intelligence Project, Memo AIM-145, Computer Science Department Report No. CS 221, June 1971. Available from the Clearinghouse for Federal Scientific and Technical Information, Springfield, Virginia 22151.

Plans:

We expect to finish soon the basic theory formation program. Most parts are completed now but have not been interfaced with each other. Running the program with small, well-structured sets of data will provide a good test for the soundness of this approach. We are confident that the program will perform satisfactorily, but we are also ready to program an attractive alternative strategy in case that is necessary. For the purpose of testing the theory formation program, the mass spectra already collected for testing the Heuristic Dendral System will be sufficient.

In the near future we expect to add more capabilities to the computer program and test it on large classes of data. For example, generalization will be programmed initially to proceed with extreme caution. In subsequent versions of the system, less cautious forms of generalization will be tried and capabilities for specification will be added. Also, the first version will be able to formulate only rules about combinations of molecular cleavages, while it is desirable to add the capability for formulating rules encompassing more complex mass spectrometric processes such as group migration. It will also be necessary to expand the library of mass spectra in order to provide adequate tests of this system. So, these data will have to be collected during this time period.

The development of data acquisition systems for mass spectrometric data is not new. The system under development at Stanford will add to the data acquisition phase an intelligent interpretation system. With

routine use of gas chromatography-mass spectrometry systems a vast avalanche of data threatens to inundate the analytical chemist. For instance during the analysis of the constituents of urine several hundred mass spectra would be recorded in the space of one hour. Clearly the sheer volume of material is beyond the interpretive power of chemists within any reasonably short period of time. One solution to this problem is to develop computer programs capable of reasoning their way through a large compilation of complex data. Heuristic Dendral is the beginning of one such program.

References:

1. J. Lederberg, "DENDRAL-64, A System for Computer Construction, Enumeration and Notation of Organic Molecules as Tree Structures and Cyclic Graphs. Part I, Notational Algorithm for Tree Structures," NASA Report CR-27029, December 15, 1964. Copies are available from the author.

2. J. Lederberg, "Topological Mapping of Organic Molecules," Proc. Nat. Acad. Sci. U. S., 53, 134 (1965).

3. J. Lederberg, "Topology of Molecules" in the National Research Council's Committee on Support of Research in the Mathematical Sciences, The Mathematical Sciences, M.I.T.Press, Boston, Mass., 1969, p. 37.

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8. A. Buchs, A. B. Delfino, A. M. Duffield, C. Djerassi, B. G. Buchanan, E. A. Feigenbaum, and J. Lederberg, "Applications of Artificial Intelligence for Chemical Inference, VI. Approach to a General Method of Interpreting Low Resolution Mass Spectra with a Computer." Helv. Chim. Acta 53 (6) 1394-1417 (1970).

9. B. G. Buchanan, A. M. Duffield and A. V. Robertson. "An Application of Artificial Intelligence to the Interpretation of Mass Spectra," in Mass Spectrometry: Techniques and Applications, Ed. G. W. A. Milne, John Wiley & Sons, New York, 1971, pp. 121-178.

c. Computer Aided Research Instrumentation

Since the last report, the major research objective has been the continued development of the concept of using dispersed computer elements to interface research instrumentation to large time-shared computer systems. The goal of this approach is to utilize dedicated, inexpensive mini-computer capabilities to satisfy real time, reflexive instrument interface and data acquisition requirements, and to buffer extracted information from a raw data stream into sophisticated analysis procedures available, without real time turn-around commitments, on time-shared large machines. To act as a test bed for these ideas and equally importantly to provide a necessary information source for advanced Dendral project and other chemical structure analysis work, the ability to acquire and reduce high resolution mass spectrometer data is being developed. A PDP-11 mini-computer is used to interface existing mass spectrometers to the ACME IBM 360/50 time-shared computer. Progress has been made in five major aspects of the problem.

1. Remote Mini-Computer Programming:

   A capability has been developed to program and control the mini-computer using the sophisticated processor, filing, and editing abilities of the time-shared machine. A basic assembler for the PDP-11 was written on the ACME machine. Programs can be written, edited, assembled, and filed on the 360/50 and then retrieved and transferred to the PDP-11 as required for execution via an interface and a minimal bootstrap loader resident in the PDP-11. Required supervisor and high resolution mass spectrum acquisition software has been written for the PDP-11 in this way. At this time only the basic assembler is available making programming for the PDP-11 fairly time consuming and requiring expert programmer support. Given the large machine capabilities of the 360/50, higher level language processors could be written for the PDP-11 thereby making programming the PDP-11 considerably easier.

2. Computer Assisted Experiment Set-up:

   Programs have been written to assist the mass spectrometer operator in setting up the instrument and computer interface to perform a mass spectrometer run. This assistance takes two forms: Prompting of operator actions to assure completion of necessary set-up procedures and interaction with data interface adjustments to minimize data loss and/or distortion. It is desirable to automate these operations as much as possible to assure consistent set-up and to make data acquisition procedures adaptive to instrument variations.

3. High Resolution Mass Spectrum Acquisition and Analysis:

Because of hardware changes within the ACME computer system a new set of interface hardware between the PDP-11 and the 360/50 had to be built. This hardware (270-Z) allows the transmission of data either way between the machines. Using primarily previous interface hardware developed for the MS-9 mass spectrometer, the new MAT 711 spectrometer was brought on-line to the PDP-11. With this hardware configuration, programs were written for the PDP-11 and the 360/50 to acquire and reduce high resolution ($M/\Delta M \geq 20{,}000$) mass spectral data. This presents a good test of mini-computer capacity in that in excess of 5×10^5 spectrum samples must be processed in 60 seconds at present and in approximately 20 seconds in the future. The initial version of the operational system performs fairly well, although given the high data rate, the need for adaptation to instrument fluctuations becomes a necessity because computer data buffer overflows can result from poor peak detection threshold settings. The PDP-11 transmits only the significant mass peak information to the 360/50 thereby reducing the data volume by approximately 95%. The 360/50 analyzes the remaining peak information to apply instrument calibration data and to derive elemental compositions for mass peaks. These are then displayed for the chemist, filed for future retrieval, or transmitted to the Dendral structure interpretation programs via a telephone line interface.

4. Instrument Control Interface:

Because of the high volume of time varying diverse-data available from a gas chromatograph/mass spectrometer (e.g. normal versus metastable spectra, variable ionization energy, etc.), not all data can be analyzed or even collected in a run. It is thus necessary to select that subset of most significance based on real time interpretive analyses and to command the instrument to operate with the proper mode and parameters to optimize data collection. Initial design work has begun for a general interface between the PDP-11 and the mass spectrometer to allow the control of selected instrument parameters such as scan initiation, sample probe temperature, and metastable spectrum acquisition.

5. Function Allocation Between Computers:

As the sophistication of analysis procedures which must be performed in real time increases along with data rates and volumes, the requirements on real time computer support increase. An initial study was made, in the context of the Dendral high resolution spectrum acquisition and analysis problem, of the desirability of using an increasing number of coordinated mini-computers versus increasing the capacity of a single processor to achieve the required computing support. The study was constrained to consider the ACME 360/50 as the larger processor alternative to the multi-mini approach. Based on initial Dendral requirements and this constraint it appears that the coordinated multi-mini approach supplies the needed computing support most economically. Given recent experience with high

resolution mass spectrum analysis, this study will be redone with better estimates of long term Dendral requirements and with no constraint on large machine selection to finalize the selection of long term approach.

An additional problem arises in considering the automated closed loop computer analysis of instrument data. One of the primary functions of the front-end processor is to extract "significant" information from the raw stream and to pass it on to subsequent analyses. It is clear that if either the definition of "significant" changes due to later analysis or if data which otherwise would be insignificant shows an instrument or experiment peculiarity which should be brought to the attention of the downstream analyzer, then valuable information may be lost unless the front end processor is "intelligent" enough to adapt to these situations. Furthermore, the quality of extracted information varies both as instrument performance varies and as signal-to-noise properties. An objective quality measure could qualify extracted data in the sense that the amount of detailed analysis time spent downstream could be adjusted based on inherent data quality hereby minimizing processor loading. This problem will be better defined and investigated in future work.

During the next report period work will continue to integrate the low and high resolution mass spectrometer data acquisition and analysis procedures. Capabilities to control instrument operation will be developed further with the goal of closed loop operation in

conjunction with the Dendral programs. A broader study of the optimum computer system configuration and function allocation design for long term Dendral needs will be undertaken.

II. Constituents of Urine

The separation of the components present in urine has been approached using an initial resolution into acidic and basic fractions. Following chemical derivatization of the acidic fraction we have been able to observe over 80 individual peaks on a gas chromatogram. This work is connected with our interest in developing computer programs for the computer interpretation of the mass spectra of biologically significant molecules and is discussed more fully in the preceding section of this report. Work with the basic fraction present in urine has concentrated on the isolation of catecholamines because of their importance in neurological systems. To date we have not been successful in the isolation of catecholamines from urine and we attribute this failure to their facile oxidation. We have, however, been successful in developing a derivatization procedure for the gas chromatographic-mass spectrometric identification of catecholamines.

An all glass silicone membrane separator has been developed for use in the direct coupling of a gas chromatograph with a mass spectrometer. Preliminary studies indicate that this apparatus provides a high transfer of material from the gas stream of the chromatograph into the mass spectrometer and no thermal fragmentation of compounds in the separator has been observed.

III. Applications of Gas Chromatography

During the past year we have constructed an automatic instrument for the analysis of phenylalanine in serum. The basic approach involves the chemical derivatization of phenylalanine in the modified injector port of a gas chromatograph which then provides the separation necessary for the identification of the derivatized phenylalanine. This system has now been completely automated and requires no technician in attendance during routine operation. For instance, sample injection, injector pyrolysis, oven temperature control and carrier gas flow are all automatically operated according to a pre-scheduled mode of operation. This technique would allow over one hundred plasma samples to be screened for phenylalanine content per day using a four-column gas chromatograph.

As a method of determining amino acid constituents of soil it is planned to obtain these compounds by solvent extraction and then derivatize their amino group. A known amount of deuterated amino acid will be added (to act as an internal standard) and the quadrupole mass spectrometer will be assigned to scan selected significant ions in the mass spectra of the amino acid derivatives. By also scanning the mass positions of the deuterated amino acid derivatives quantitation will be introduced into the analysis.

Gas chromatography was also employed as the analytical tool in the construction of a technique for the determination of the amount of cyclohexyl amine in aqueous solutions of sodium cyclamate. Detection limits in the 10^{-11} gram range were achieved using this method. The rate of hydrolysis of sodium cyclamate to cyclohexylamine in acid solution was determined using this procedure.

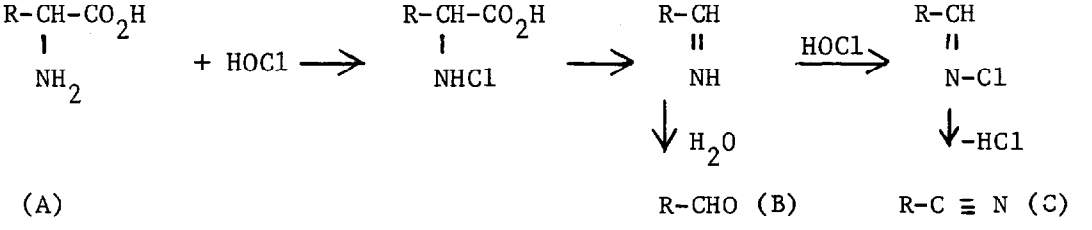
IV. Clorination of DNA Bases

Chlorination studies using aquoeus hypochlorous acid and the bases present in DNA has continued. Work has centered around a clarification of the chemistry involved in the reaction of hypochlorite solution with cytosine. We have been able to identify pure crystalline compounds from this reaction depending upon the amount of hypochlorous acid used. These compounds are N-chloroamines and further work is in progress to elucidate the chemistry behind the reaction of hypochlorite solution with the other bases found in DNA. It is hoped that this study will provide some clues to the manner in which chlorine kills bacteria and whether this involves attack on the DNA of the bacteria by chlorine.

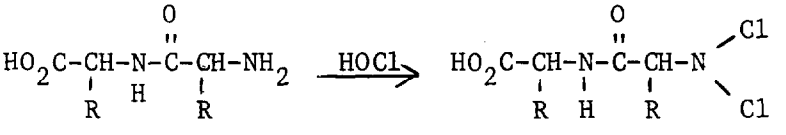
The isolation of 4-N-chlorocytosine and its reaction with one equivalent of hydrobromic or hydroiodic acids results in a new, simple and direct route to the chemical preparation of 5-bromo- and 5-iodocytosine, respectively.

Another possible cause of the lethal power of hypochlorous acid on bacteria could be due to the cessation of amino acid synthesis in the cell. In order to examine the chemical implications of this concept we have examined the reaction of hypochlorous acid with amino acids. To date phenylalanine, tyrosine,and glutamic acid have been examined. We observed that the product from these reactions were good yields of the corresponding nitriles formed by the following reaction sequence. Previous workers using a much higher pH (8.0 compared to pH 3 in our studies) report the isolation of aldehydes (B) in good yield. Under the

the conditions used we find the aldehydes to be only a minor component of the reaction. In addition chlorination of the aromatic ring of tyrosine occurs when it is treated with hypochlorous acid. This had not previously been reported.



The chemical reaction of di- and larger peptides with hypochlorous acid does not seem to have been studied previously in any detail. We have observed the formation of the N,N-dichloro compound (D) from several dipeptides examined. The chemistry of this new class of compound is being examined with reference to its possible hydrolysis under mild conditions to the amino acid of the carboxyl end of the original peptide.



dipeptide (D)

V. Mass Spectrometry of Organic Compounds

We became interested in the mass spectra of promazine sulfoxide (a member of the phenothiazine group of tranquilizers) during an investigation of its metabolism in sheep. The mass spectra of the sulfoxide of promazine contained a distinct feature not observed in the mass spectrum of promazine itself. This difference involved the transfer of two hydrogen atoms and we have commenced the labeling of the side chain of promazine sulfoxide with deuterium in order to better understand the precise mechanism involved in this mass spectrometric fragmentation.

Another study in the field of mass spectrometry has been concerned with the fragmentation behavior of a series of pimelic acid derivatives. From extensive deuterium labeling we hope to be able to discover to which functional group the hydrogen atom preferentially moves in the McLafferty rearrangement process.

New Publications:

L. G. Brookes, M. A. Holmes, I. S. Forrest, V. A. Bacon, A. M. Duffield, and M. D. Solomon. "Chlorpromazine Metabolism in Sheep II. In Vitro metabolism and preparation of ^{3}H-7-Hydroxychlorpromazine." (Submitted to Agressologie 1971.)

M. D. Solomon, W. E. Pereira and A. M. Duffield. "The Determination of Cyclohexylamine in Aqueous Solutions of Sodium Cyclamate by Electron-Capture Gas Chromatography." Analytical Letters 4(5), 301-304 (1971).

W. E. Pereira, B. Halpern, M. D. Solomon and A. M. Duffield. "Electron-Impact Promoted Fragmentation of Alkyl-N-(1-Phenylethyl)-Carbamates of Primary, Secondary and Tertiary Alcohols." Organic Mass Spectrometry 5, 157-169 (1971).

B. Halpern, W. E. Pereira, Jr., M. D. Solomon and E. Steed. "A Rapid and Quantitative Gas Chromatographic Analysis for Phenylalanine in Serum." Anal. Biochem. 39, 156 (1971).

A. M. Duffield, W. E. Reynolds, D. A. Anderson, R. A. Stillman, Jr. and C. E. Carroll. "Computer Recognition of Metastable Ions." Presented at the Nineteenth Annual Conference of Mass Spectrometry and Allied Topics, Atlanta, Georgia, May 1971.

VI. Analysis of Natural Products by Mass Spectrometry

During the past year research has continued on the structural analysis by mass spectrometry of natural products isolated from plant, animal and marine sources. A list of publications resulting from this experimentation follows:

G. Eadon, J. Diekman and C. Djerassi. "Application of Ion Cyclotron Resonance to the Structure Elucidation of the C_3H_6O. Ion Formed in the Double McLafferty Rearrangement". J. Am. Chem. Soc. 92, 6205 (1970).

R. T. Gray, J. Diekman, G. L. Larson, W. K. Musker and C. Djerassi. "Mass Spectrometry in Structural and Stereochemical Problems - CLXXXIX: Electron-Impact Induced Fragmentations and Rearrangements of Alkoxycyclohexanol Trimethylsilyl Ethers and Alkoxycyclohexyl Trimethylsilanes." Org. Mass Spectrometry 3, 973 (1970).

G. G. Smith and C. Djerassi. "Mass Spectrometry in Structural and Stereochemical Problems - CXCIX. Contrasting Fragmentations of Singly- and Doubly-Charged o-, m- and p-Hydroxyalkylphenones and their Trimethylsilyl Ethers. Org. Mass Spectrometry 5, 505-523 (1971)

R. J. Liedtke, A. M. Duffield and C. Djerassi. "Mass Spectrometry in Structural and Stereochemical Problems - CXCIII: On the Combined Hydroxyl and Water Loss in Nitrophenylhydrazones." Org. Mass Spectrometry 3, 1089 (1970).

VII. Mariner Mars 1971 Orbiter Photography

The picture processing capacity described in Technical Report IRL 1123 has been implemented in support of the Mariner spacecraft presently orbiting Mars. The system was developed and tested utilizing Mariner Mars '69 data and simulation data designed to determine its capacity to isolate image brightness changes of as little as 5%. The "W" cloud formation thought to have appeared in some of the Mariner Mars '69 Far Encounter images was quite clearly revealed by image differencing utilizing the system.

The above mentioned report describes, in detail, the geometric and photometric normalization and alignment procedure necessary for automated picture differencing. Various digital filtering and enhancement techniques have also been developed in support of the picture differencing work. These techniques are also helpful in aiding with the visual inspection of the images. Data tapes containing the raw TV images (PTV-EDA) and the geometrically photometrically corrected TV images (RDR) are bing sent to us as they become available at the Jet Propulsion Laboratory. Also received is supplementary data (TQL-SEDR) describing the spacecraft conditions under which the images were recorded.

Figure 1 is an example of a picture differencing operation. The area in question is along the edge of the South Polar Cap at about -87° latitude, 20° longitude (West). The upper left image was acquired on orbit 28, the upper right three days later on orbit 34. Both images

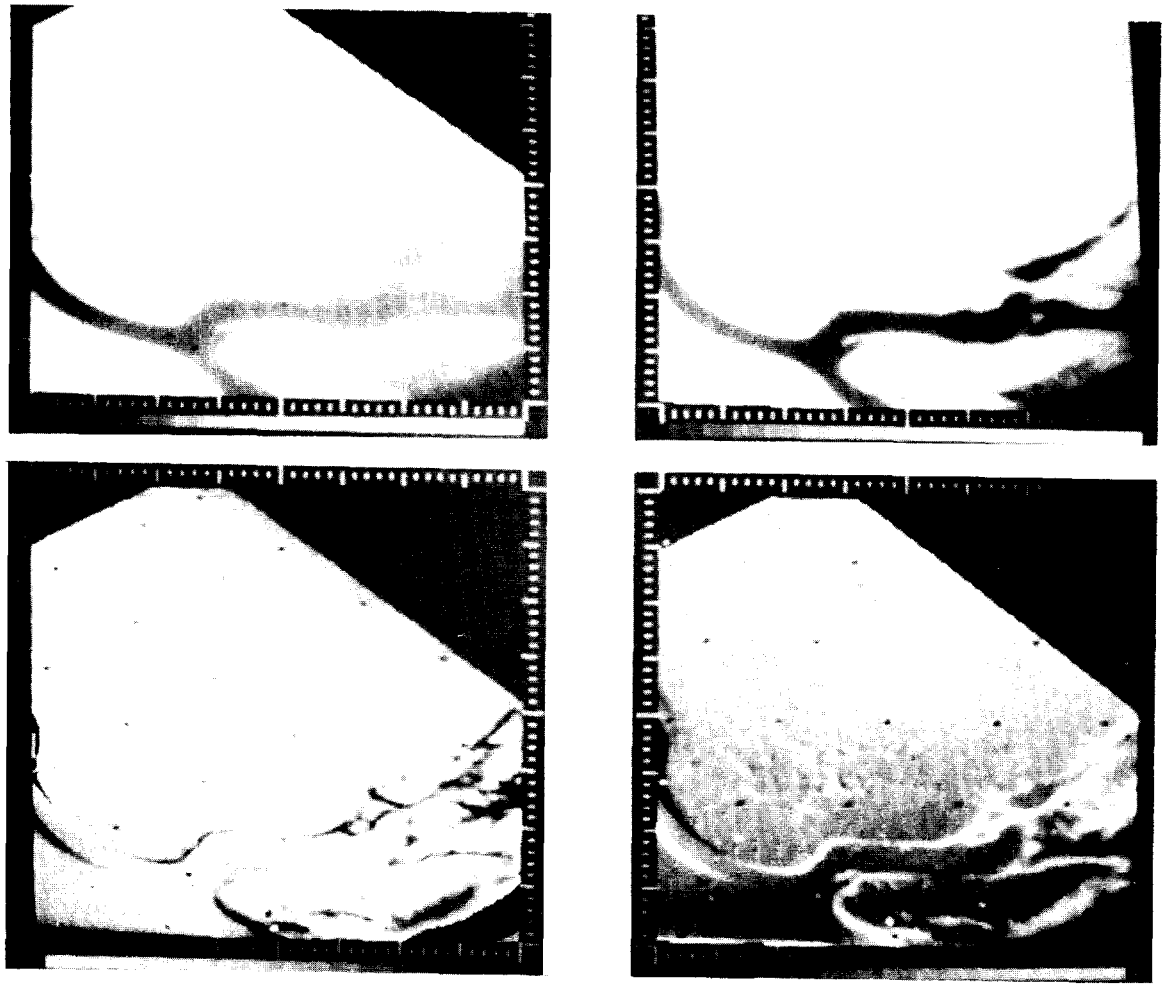


FIGURE 1.

were transformed to the same orthographic projection and then the upper right image was registered to the other using the correlation techniques described in the above mentioned report.

The lower left image is the difference image gotten by subtracting the upper right from the upper left. The lower right image is the negative of that difference (upper left from upper right). The bright and dark region on the difference images represent changes in the original images. However, some apparent differences appearing along the border are anomalies and are due to poor image registration along the edges.

Special enhancement operations are also being performed. Figure 2 shows two views of Phobos (innermost moon) with each view subject to two enhancements. The upper image was taken on orbit 43, the lower on orbit 48.

Work of the type described above is intended to continue through the primary mission, the extended mission, and the post-mission analysis.

The work is being carried out jointly with the Stanford Artificial Intelligence Laboratory of the Computer Science Department. The Stanford Linear Accelerator Center (SLAC) has provided hardware support in the form of two 70 mm film viewers; one installed at Jet Propulsion Laboratory, the other at the Artificial Intelligence Laboratory.

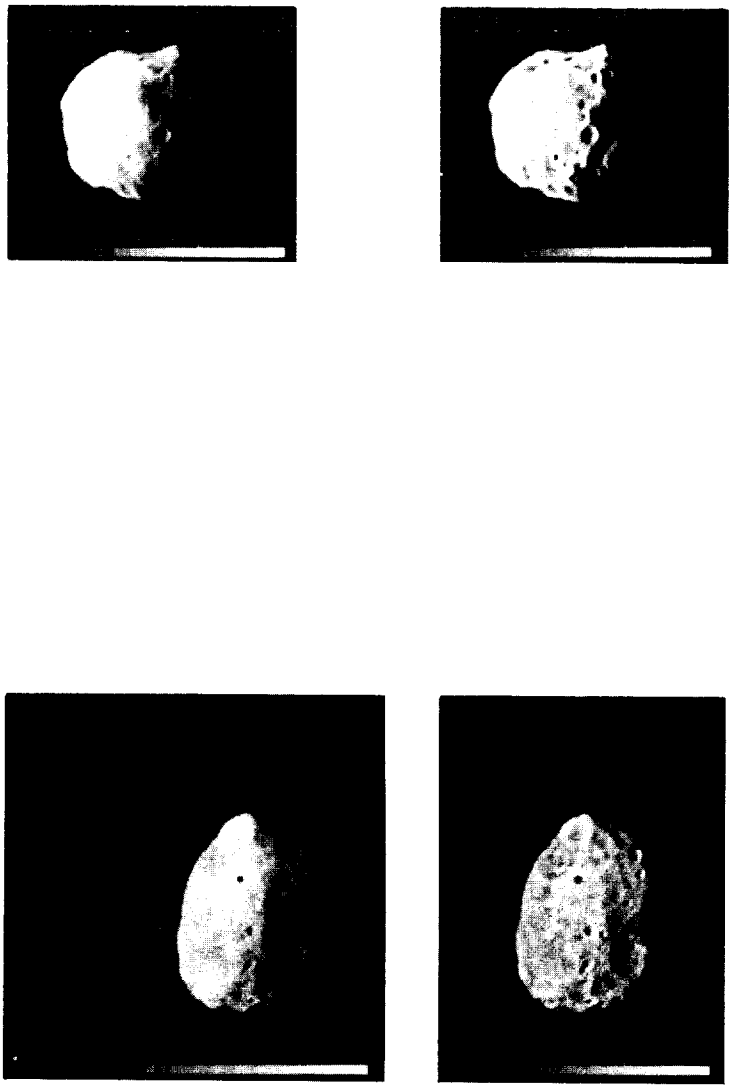


FIGURE 2.

VIII. Viking Lander Imagery Investigation

Activity during the past year has fallen into two general areas:

1. Participation in the development of camera and mission operation specifications; and, recently,
2. Commencement of computer analysis of prototype and projected lander camera imagery data.

The specification work has been concerned with camera details and with requirements for image reconstruction hardware and data manipulation software.

The computer effort has been devoted to problems associated with the extraction of near-field ranging information from lander camera stereo image pairs. This latter work, which has used the facilities of the Stanford Artificial Intelligence Laboratory, is described below.

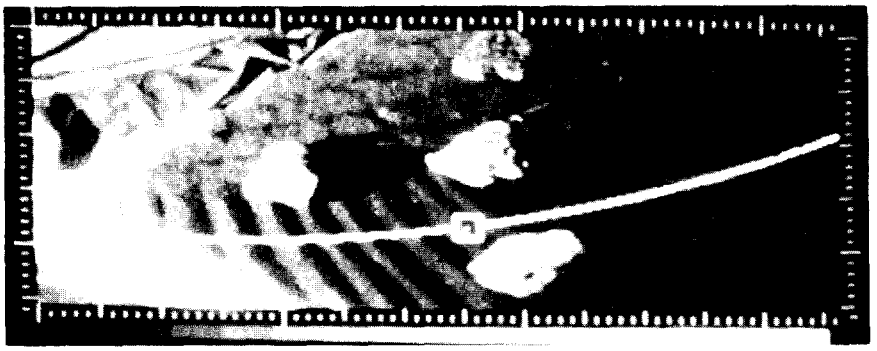
Figure 3 illustrates a stereo pair of images recorded of a portable terrain model by a lander camera prototype. The figures displayed in this and the following illustrations are reproductions of Polaroid pictures taken of the output of a computer driven video-synthesizer. The upper and lower images represent left and right views, respectively, of the model as recorded by a single lander camera prototype located at two different positions 100 cm apart, approximately 135 cm above the model and at roughly 100-200 cm horizontal range. The model consists of an undulating surface of dark sand upon which have been placed four conspicuous rocks and some smaller pebbles. Since the horizontal range

Figure 3. A stereo pair of images recorded of a portable terrain model by a single lander camera prototype located at two different positions. Upper image left view; lower image right view.

is comparable to the inter-camera displacement, henceforth called the "baseline", the two views of the scene appear appreciably different. It has been determined experimentally that it is impossible to visually fuse such disparate images, a prerequisite to visual depth perception.

Figure 4 indicates the same pair of images upon which have been overlayed two smooth curves. These curves have been constructed as follows. A plane, henceforth referred to as the "camera-centers plane", is defined to contain the two effective camera-center positions. This plane has been rotated about the baseline until it passes through the selected point of interest in the left view at the center of the prepositioned box located directly beneath the central rock. This plane projects into the left and right camera views as the curves shown. The fact that the plane projects as a curve rather than a straight line is a consequence of the scanning geometry of the facsimile camera and of the associated display format. Specifically, the camera scans the scene, point by point, in uniform polar and azimuthal steps. The display format is linear in polar and azimuthal angles, and hence represents a linear mapping of the original recording. The lowest point in each of these curves corresponds to an azimuthal viewing direction perpendicular to the baseline at the camera location in question. Scene points lying on the camera-centers plane and common to both views must necessarily lie on the projections of this plane in each image.

Figure 5 illustrates the same image pair overlayed with image point location boxes, used in the scene ranging mode. The box in each view has been visually positioned to a scene point that is common to both



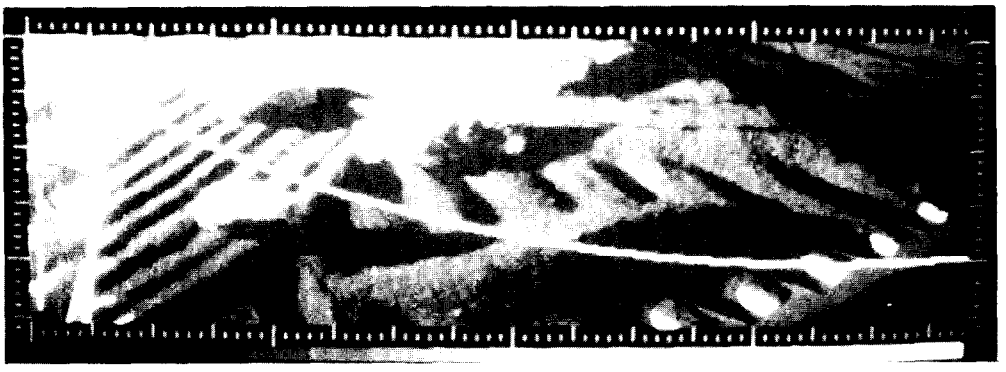


Figure 4. Overlay of a projection of the camera-centers plane onto the stereo images.

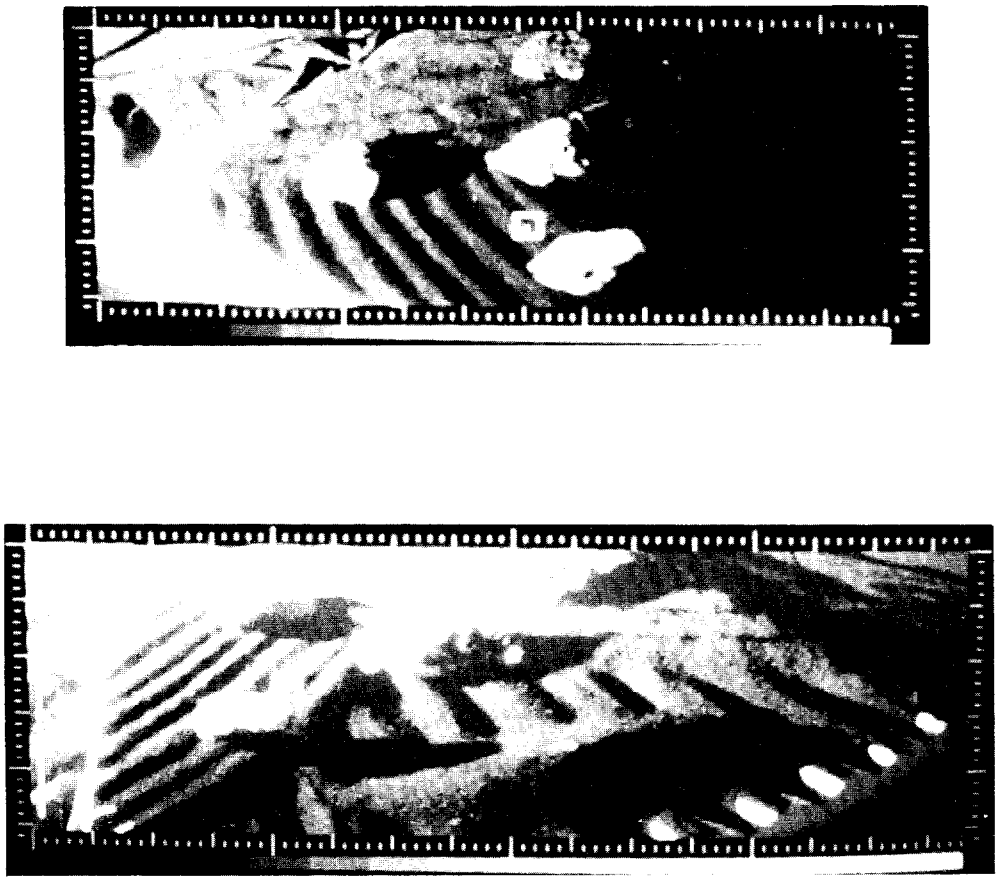


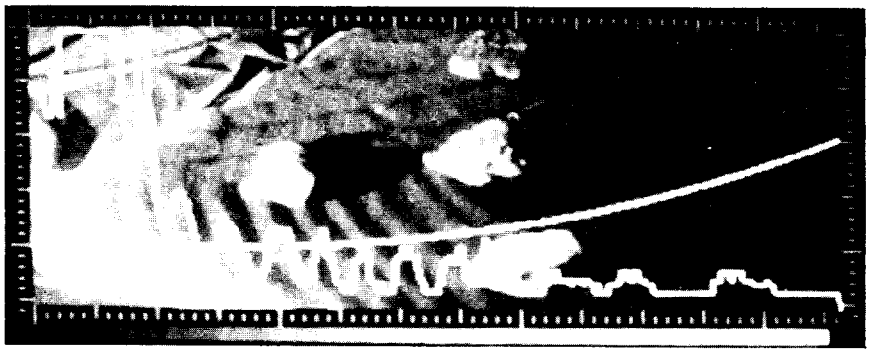
Figure 5. Stereo images overlayed with image point location boxes in the scene point ranging mode.

views and is to be ranged. The positioning procedure is as follows. The box is first interactively centered about a point of interest in the left camera image. A camera-centers plane is then tilted to contain this pointing direction. The physical point of interest in the right view now must lie on the projection of the plane in that image. Thus, only a single degree of freedom remains for the definition of the corresponding box location in the righthand view. This latter parameter has been arbitrarily chosen to be the x-coordinate of the point, in image coordinates. The location of the matching point in the right view is determined visually. The box is then brought to position by the adjustment of the x-coordinate. The associated value of the y-coordinate of the point is then immediately evaluated.

Once the corresponding pointing directions in the two scenes have been established, we have sufficient information to evaluate the spatial location of the selected point relative to the cameras. The ranging information is promptly computed and recorded and/or displayed.

Following development of the above programs we moved on to a familiarization study of some of the characteristics of the unique data format, preparatory to investigating automation of ranging and contour map production.

A first step in this process is indicated in Fig. 6, which contains additional overlays to those discussed above. The oscillatory curves appearing in these views represent plots of the scene intensity scanned along the camera-centers plane and parameterized linearly in the



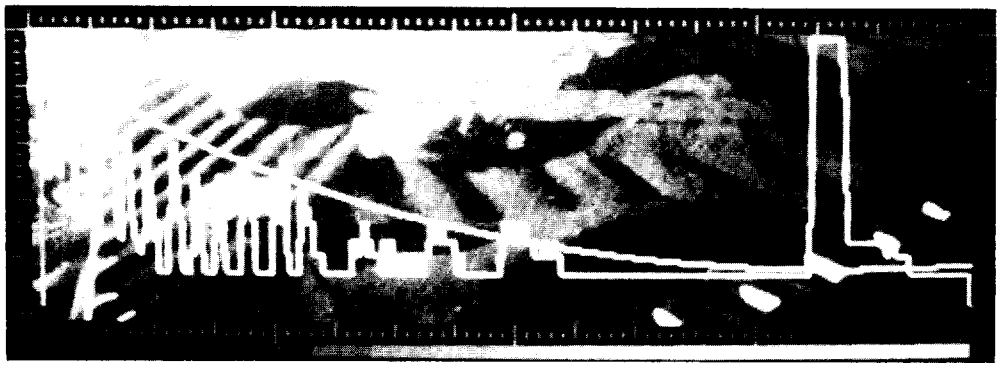


Figure 6. Overlays of the scene point intensity along the camera-centers plane as a function of the x-coordinate of the image point.

x-coordinate. It will be noted that the lines of constant phase for the sand ripples running across the lower lefthand portion of the left view are roughly orthogonal to the baseline. These same waves, when viewed from the righthand camera position are observed obliquely and consequently exhibit foreshortened projected wavelength. The relative distortions associated with the grossly different perspectives of the two views pose special picture point correlation problems for automation of ranging.

Figure 7 illustrates a remapping of the intensity plots of Figure 6 into a format that would be more amenable to a one-dimensional correlation of data from the two scenes. The intensity curves in Figure 7 have been obtained by transforming the curves in Figure 6 to the appearance they would have if recorded by a camera located at the mid-point between the left and right camera positions, under the assumption that the scene is perfectly flat, horizontal, and at the nominal value of observed relative elevation. The horizontal scale has been changed to utilize the full width of the screen. The mapping between the scene and the image now is nonlinear and the mutual interrelation no longer is as readily discernable as in the previous illustrations. It is evident, however, that one-dimensional picture point correlation would be more readily conducted in this transformed image intensity space than in the initial space.

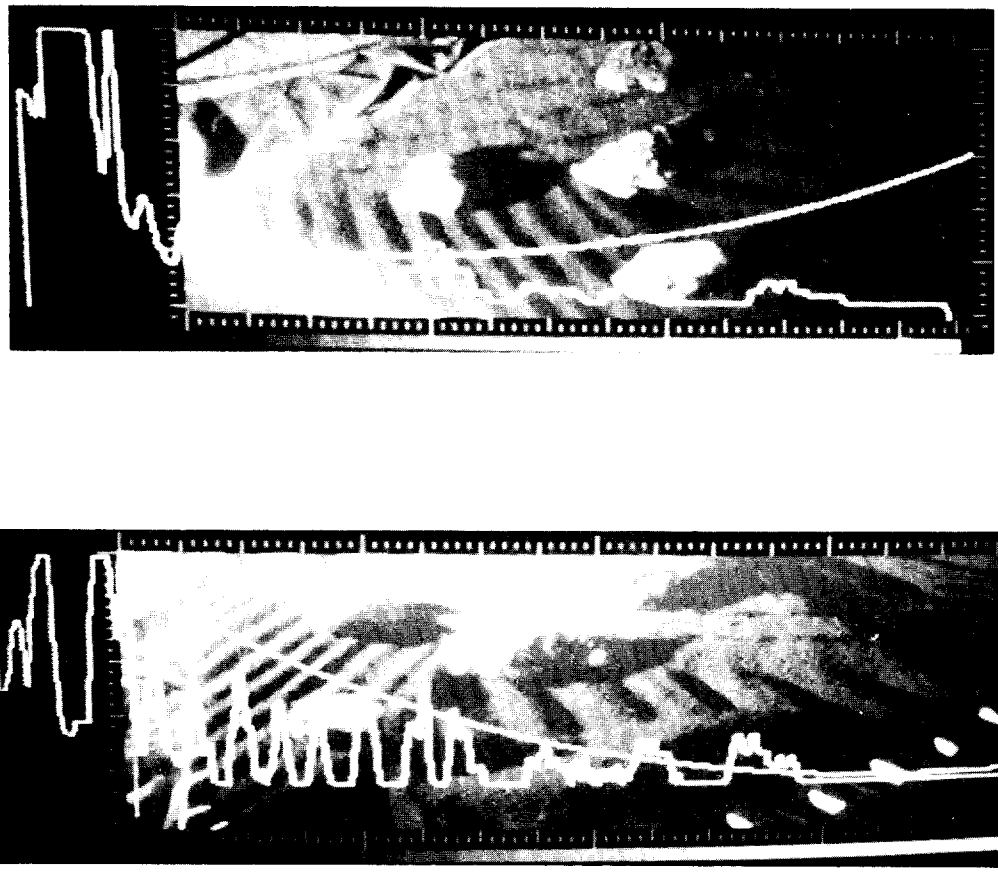


Figure 7. Overlays of transformed scene point intensities.

We do not plan to proceed further along the above lines. Rather, we are considering a more general and powerful approach toward the development of near-field automated ranging. The proposal is to explore the portion of the 3-space mutually accessible through the left and right windows, by correlation of left and right imagery data over a variously tilted and positioned probing planar patch. Once it has been determined, by prescribed correlation criteria, that we have succeeded in "landing" on a surface, we can "crawl" over the surface in any manner desired, accumulating ranging information as we go. Elevation contour lines could for example be generated by instructing the probe to explore at fixed elevation. An automated contour map could thus be constructed.

We also are commencing various image transformations directed both toward compensating for inherent projective distortions and toward facilitating the comparison both of images taken from the same camera under different recording conditions and of images taken from the two different lander camera positions.

IX. Cell Separation

While this work was initiated by the subject grant most of the support is now coming from NIH Grant GM 17367. The work has obvious applications in the medical field as well as for exobiology.

A. High Speed Fluorescent Cell Sorter

This unit is designed to measure the fluorescence of cells in a jet of liquid, break up the jet into uniform drops and collect the drops in a series of containers, with all drops containing cells with similar fluorescent characteristics collected in the same container.

As previously reported, the jet has been modified by incorporation of a modified Crossland-Taylor sheath flow system which confines the cells to the central portion of the stream and thus minimizes variation in cell output as a function of cell position. The system sensitivity has been increased greatly by substitution of a medium power argon ion laser for the mercury arc.

Improvements made during the past year have made it possible to increase efficiency of separation by a factor of about two by reducing the uncertainty regarding the number of drops which must be deflected to be certain of deflecting the desired cell. A number of other modifications have been made resulting in a system of improved speed, reliability, reproducibility and ease of operation.

Much of the recent instrumental effort has been devoted to design and construction of a multichannel cell separator, able to separate on the basis of signals from fluorescence in two different spectral ranges. This unit is essentially completed and is undergoing preliminary testing. In order to take full advantage of the capabilities of this unit a two parameter pulse height analyzer has been incorporated into the system.

Also incorporated into the system is a third photoelectric channel which uses low angle forward scattering to provide signals from all cells in the stream. This signal is roughly proportional to cell volume, and will be useful in determining flow rates and relative number of fluorescent cells. It is also needed to permit optimum use of an anti-coincidence circuit we have designed. This unit, which substantially increases the purity of separated cell fractions, deflects groups of drops which contain both a desired cell and one or more undesired cells into a separate container, and thus prevents contamination of the desired cell fraction with undesired cells.

The scatter unit can be adapted for absorption measurements, if desired, by simple mask changes, allowing us to experiment with a wide assortment of absorption stains as separation criteria.

In order to optimize system performance we have undertaken studies aimed at determining the relationships between operating conditions and variations of signals from cells with equal fluorescence output. Minimum coefficient of variation so far has been observed with a suspension of very uniformly fluorescent beads kindly supplied to us by Los Alamos

Scientific Laboratory. In our system these beads had a coefficient of variance (the ratio of standard deviation to the mean) of less than 9%. This appears to be low enough for any separations we presently envision. Our standard test objects, chicken erythrocytes stained with quinacrine mustard, have a coefficient of variation of about 15%, indicating remarkable uniformity for a biological specimen.

As a result of the system improvements the value of the instrument for biological experiments has been greatly increased. Papers by M. Julius, Masuda and Herzenberg*, and by Herzenberg**, describe some of the experiments involving use of immunofluorescent techniques to separate cells with particular antigen binding characteristics. In one series of of experiments we were able to separate virtually all cells capable of giving rise to antibody production against human serum albumin (HSA or Keyhole Limpet Hemocyanin (KLH). Suspensions of spleen cells from a mouse primed with HSA were incubated with HSA and then with fluorescein labelled goat anti-HSA, and the fluorescent cells were separated. These cells were unable to give rise to anti-HSA production when injected into an irradiated host alone, but when supplemented with thymus derived cells also unable to give antibody response alone they proved to contain nearly all of the precursors to antibody producing cells. Similarly the fraction of spleen cells stained with a fluorescent anti-immunoglobulin reagent proved to contain the great majority of precursors of cells

* M. Julius, T. Masuda and L. A. Herzenberg. "Isolation of Functional Antibody Forming Cell Precursors Using a Fluorescence Activated Cell Sorter." (In preparation)

** L. A. Herzenberg in "Immunogical Intervention", Jonathan Uhr and Maurice Landy, eds. Academic Press. 1971.

capable of producing antibodies to sheep red cells. These findings are in accordance with the hypothesis that the precursors to antibody producing cells carry immunoglobulin and specific antigen binding markers, and require thymus derived (T) cells to enable them to differentiate to the actual antibody producing cells. It appears that the separator may be able to play a major role in investigating the immune system and its role in such clinical problems as transplant rejection.

These results constitute the first successful enrichment of specific functionally active antibody forming cell precursors.

A recent most exciting result using the machine has been the direct demonstration that cooperator cells may have IgM on their surfaces. Such cells were obtained by indirect fluorescent staining for IgM of spleen cells from animals injected previously with sheep red blood cells as antigen. The stained live cells were separated. These cells added to another portion of the same spleen cell suspension which had been depleted for cooperator thymus-derived (T) cells fully restored the cooperator activity. That is, the T cell depleted population did not give rise to antibody forming cells while this population plus added IgM stained and separated cells did. This then may be the first direct demonstration that cooperator T cells have surface IgM.

Another area of current interest is research into the relationships between uptake of conconavalin A by cell surfaces and cell characteristics. This protein, derived from jack bean meal, has a special affinity for certain carbohydrate groups on cell surfaces, particularly for α D

glucopyranosyl residues. For similar cells the amount of Con A taken up is probably proportional to cell surface area. However there are reports that it shows special affinity for virus transformed cells and also for cells in the process of mitosis. It may also show different affinities for different functional types of cells, so that relative Con A binding might be used to separate such types.

We have been able to incorporate fluorescein in the Con A molecule and reacted this fluorescent protein with cell populations from mouse bone marrow, thymus and spleen. When kept cold the cells do not clump. There are measurable differences between the various populations but we have not determined yet how much of the differences are the result of size as compared to qualitative differences in cell surface carbohydrates. We have, however, measured mouse thymocyte binding at various ages and found significant differences, with maximum binding apparently occurring in the juvenile (4 wks) compared to newborn or adult (78 wks). This indicates that functional differences (or position in the mitotic cycle) do affect Con A binding. Quantitative binding curves have been obtained over a 2^{18} fold range of Con A concentration. We have treated human leucocyte suspensions with Con A and found two distinct peaks in the pulse height distribution. The treated cells were separated. The deflected fraction (consisting mainly of cells giving higher amplitude signals) was about 90% granulocytes, the undeflected fraction about 90% lymphocytes. The original suspension was about 75% granulocytes and 25% lymphocytes.

A series of similar proteins react with different carbohydrate moieties, so if the Con A proves able to distinguish between functional types a completely new parameter for cell separation will be available. In addition the fact that Con A binding appears to be affected by transforming virus and by mitosis strongly suggests that it or some of the similar proteins may be of use in identifying and separating neoplastic (tumor) cells from the general cell population.

Studies using the binding of Rh^+ antibodies in a sandwich with fluoresceinated goat anti-human gamma globulin to detect Rh^+ blood cells are currently underway. This reaction appears to work well, producing tagged cells with relatively uniform signals and good signal to noise ratio. The technique holds promise in detecting and quantitating leakage of Rh^+ cells to an Rh^- mother's blood during pregnancy and delivery since we have been able to detect Rh^+ cells in a frequency of 10^{-4} to 10^{-5} the number of Rh^- cells, i.e. 1 Rh^+ cell per 10^4-10^5 Rh^- cells.

Other work has shown that the instrument can detect reticulocytes stained with acridine orange and thus may be useful for providing automated reticulocyte counts. It is faster than the presently used manual methods, and would have the advantage that many more cells could be processed, reducing the relative statistical variation. The counts recorded so far on a variety of samples correspond roughly to those obtained manually on the same samples in the clinical laboratory. Work to determine the advantages and disadvantages of the method is continuing.

Acridine orange can also be used to stain human leucocytes. The ratio between the red and green fluorescence of these cells after staining appears to vary depending on cell type. We have conducted some preliminary experiments in this region with our single channel instruments, and Melamed and Bio Physics Inc. have demonstrated the differences between three groups of such cells, tentatively identified as lymphocytes, monocytes and granulocytes on a two channel analytical instrument incapable of separation. They also have found that the apparent lymphocyte fraction seems to vary with the state of health of an individual. Such variations are difficult to interpret with only an analytical instrument. Separating cells giving specific intensity signals would allow identification. We intend to explore this area intensively when the dual channel separator is in operation.

B. High Speed Volumetric Cell Sorter

Work on this unit has been given a relatively low priority, primarily because of the exciting results obtained with the fluorescent separator. A report describing the instrument and its operations has been published in The Review of Scientific Instruments.*

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* J. T. Merrill, N. Veizades, H. R. Hulett, P. L. Wolf, and L. A. Herzenberg. "An Improved Cell-Volume Analyzer." Rev. Sci. Instr. 42, 8, pp. 1157-1163 (Aug. 1971).

Publications

1. W. A. Bonner, H. R. Hulett, and L. A. Herzenberg. "Highspeed Sorting of Fluorescence Labeled Cells." Fed. Proc. 30, 699 abs. 1971

2. L. A. Herzenberg. Chairman, Conference Session, "Fluid Transport Methods", Engineering Foundation Research Conference on Automatic Cytology, New England College, Henniker, New Hampshire, July 26-30, 1971.

3. L. A. Herzenberg and R. G. Sweet. "Fluorescence Activated Cell Sorting", presented at Engineering Foundation Research Conference on Automatic Cytology, New England College, Henniker, New Hampshire, July 26-30, 1971.

4. L. A. Herzenberg, T. Masuda, and M. Julius. Invited paper on Symposium on Thymus and Bone Marrow Cells in the Immune Response, Annual Meeting of the American Society of Hematology, San Francisco, Dec. 4, 1971.

5. L. A. Herzenberg. Invited participant in symposium on Cell Purification by use of Surface Antigens and Receptors, Midwinter Conference of Immunologists, Asilomar, California, Jan. 22, 1972.

6. L. A. Herzenberg, R. G. Sweet, M. Julius, T. Masuda, and R. A. Merker. Invited paper, "Fluorescent Activated Electronic Cell Sorting in Immunology", to be presented at Biophysical Society Annual Meeting, Toronto, Canada, Feb. 19, 1972.

7. W. A. Bonner, H. R. Hulett, R. G. Sweet and L. A. Herzenberg. "Fluorescence Activated Cell Sorting." Rev. Sci. Inst. (In press, scheduled for publication March 1972)

C. REPORTS AND PAPERS

This section lists papers and reports not referred to in preceding sections of the Program Resume.

REPORTS

1. Cytotoxicity Assay Automation Final Report. Contract 69-2064 NIH Transplantation Immunology Branch. Technical Report No. IRL 1129 (1971).

PUBLICATIONS

1. W. E. Reynolds, V. A. Bacon, J. C. Bridges, T. C. Coburn, B. Halpern, J. Lederberg, E. C. Levinthal, E. Steed and R. B. Tucker. "A Computer Operated Mass Spectrometer System." Analytical Chem. 42, 1120 (1970).

2. B. Halpern, W. E. Pereira, Jr., M. D. Solomon and E. Steed. "A Rapid and Quantitative Gas Chromatographic Analysis for Phenylalanine in Serum." Anal. Biochem. 39, 156 (1971).

3. Y. M. Sheikh, A. Buchs, A. B. Delfino, G. Schroll, A. M. Duffield C. Djerassi, B. G. Buchanan, G. L. Sutherland, E. A. Feigenbaum and J. Lederberg. "Applications of Artificial Intelligence for Chemical Inference-V. An Approach to the Computer Generation of Cyclic Structures. Differentiation Between All the Possible Isometric Ketones of Composition $C_6H_{10}O$. Org. Mass Spectrometry 4, 493 (1970).

4. E. A. Feigenbaum, B. G. Buchanan, and J. Lederberg. "On Generality and Problem Solving: A Case Study Using the Dendral Program," in Machine Intelligence 6, B. Meltzer and D. Michie (eds). Edinburgh University Press, 1971.

5. G. Buchanan, J. Lederberg. "The Heuristic Dendral Program for Explaining Empirical Data." To be presented at the 1971 Congress of the International Federation of Information Processing Society (August, 1971) and published by North Holland Publishing Co. (1971) in press.

6. J. Cymerman Craig, W. E. Pereira, Jr., B. Halpern, J. W. Westley. "Optical Rotatory Dispersion and Absolute Configuration -XVII α-Alkylphenylacetic Acids." Tetrahedron 27, 1173 (1971).

7. W. E. Pereira, Jr. and B. Halpern. "The Steric Analysis of Aliphatic Amines with Two Asymmetric Centers Via Gas Liquid Chromatography of Diastereoisomeric Amides." Submitted to Aus ralian Jour. of Chem. (1971).

8. A. M. Duffield and O. Buchardt. "Thermal Fragmentation of Quinoline and Isoquinoline N-Oxides in the Ion Source of a Mass Spectrometer." Submitted to Acta Chemical Scandanavica (1971).